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Original Article

Ad-HGF induces autophagy in hypoxia-injured H9c2 cardiomyocytes via activating NF-κB signaling

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Abstract: Autophagy at early stage of hypoxia has been demonstrated to play a vital role in protecting myocardium. Increasing evidences suggest that transcription factor NF-κB is involved in autophagy. Furthermore, our previous studies have confirmed that adenovirus carrying the HGF gene (Ad-HGF) could protect the heart against myocardial ischemia. However, the relationship among Ad-HGF, autophagy and NF-κB signaling in myocardial infarction (MI) is still unknown. Therefore, we aimed to investigate whether Ad-HGF could regulate autophagy through NF-κB signaling in an *in vitro* hypoxia model. Autophagy was observed by transmission electron microscope and Ad-mRFP-GFP-LC3 infection. Protein expression levels of LC3-I, LC3-II, Beclin-1, p62, NF-κB p65 and phosphorylated NF-κB p65 were analyzed by western blot or immunofluorescence. Notably, we found that autophagy in H9c2 cells enhanced at early stage after hypoxia. Ad-HGF treatment dose-dependently promoted autophagy in hypoxia-injured H9c2 cells, while HGF receptor inhibitor, SU11274 yielded opposite effect. What's more, NF-κB signaling was activated by Ad-HGF in a dose-dependent manner, and the NF-κB inhibitor PDTC could efficiently inhibit the activation of autophagy induced by Ad-HGF. Taken together, our findings demonstrated that Ad-HGF promoted autophagy in hypoxia-injured H9c2 cells and the protective effect of adaptable autophagy was mediated by activating NF-κB signaling, which suggested a new potential pathway of HGF protecting myocardium from hypoxia.

Keywords: Ad-HGF, hypoxia, autophagy, NF-κB, H9c2 cell

Introduction

Autophagy is an intracellular degradation process in which cytosolic proteins and organelles are confiscated into autophagosomes and degraded by lysosomes [1]. Since its large capacity, autophagy has contributed to maintain organelle functions and protein quality by degrading damaged organelles and protein aggregates. However, excessive degradation of organelles and essential proteins may cause bioenergetic failure and eventually organ dysfunction. Numerous studies have revealed that autophagy is activated and has been noted for its defensive roles in neurodegenerative diseases, including Parkinson's, Huntington's and Alzheimer's diseases [2, 3]. In addition, accumulating evidence has indicated that autophagy is upregulated in various cardiovascular diseases, including myocardial ischemia, ischemia/reperfusion and heart hypertrophy [4, 5]. It is still controversial whether activation of autophagy in cardiomyocyte injury may present a mechanism of cell death or may be a rescue mechanism activated after the injury. Nevertheless, adaptable autophagy at early stage of hypoxia has been demonstrated to play a vital role in protecting myocardium both *in vivo* and *vitro* [5, 6].

Hepatocyte growth factor (HGF) is a multifunctional cytokine that exerts considerable biological effects including morphogenesis, stem cell maintenance and immunomodulation etc. [7]. It has been proposed as a modulator of cardiac repair of damage due to the pleiotropic effects elicited by c-Met receptor stimulation. Our precious studies have demonstrated that Ad-HGF plays an indispensable role in the cardiomyocyte regeneration, neovascularization and heart function repair after MI [8, 9]. However, little evidence has shown that the protective effect of Ad-HGF is related with the myocytes adaptable autophagy after MI.

Nuclear factor kappa-B (NF-κB) is a key regulator of inflammatory and survival pathways, which is thought to be an intracellular messenger that transmits the gene induction signal from the cytoplasm to the nucleus [10]. Our previous evidences imply that NF-κB activation caused by p27 haplo-insufficiency protects injured heart during the early stage post MI [11]. Moreover, NF-κB-mediated induction of autophagy in cardiac ischemia/reperfusion injury has been reported [12]. However, the relationship between NF-κB and autophagy is still a matter of debate. And the relationship among HGF, autophagy and NF-κB signaling has never been elucidated before. Thus, in the present study, we set out to investigate whether Ad-HGF could regulate autophagy through NF-κB signaling in an *in vitro* hypoxia model.

Materials and methods

Antibodies and reagents

The following antibodies were purchased from Abcam: p62 (ab91526), rabbit anti-mouse IgG H&L (HRP) antibody (ab6728) and goat anti-rabbit IgG H&L (HRP) antibody (ab6721); from Cell Signaling (USA): LC3A/B (#4108), Beclin-1 (#3495), p-NF-κB p65 (Ser536) (#3033), NF-κB p65 (#8242) and GAPDH (#5174); from Jackson ImmunoResearch (USA): FITC labeled secondary antibody. HGF was purchased from PeproTech (PeproTech, USA). SU11274 was purchased from Selleck Chemicals (USA). Pyrrolidine dithiocarbamate (PDTC) was purchased from Sigma (USA). Ad-mRFP-GFP-LC3 was purchased from HanBio Technology Co. Ltd, China. Replication-deficient adenovirus carrying the HGF gene (Ad-HGF) was constructed as previously described [8, 9].

Cell culture and hypoxia

H9c2 cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Wisent Inc., Montreal, QC, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/ml of penicillin and 100 U/ml streptomycin and 5% CO_2 at 37°C in a humidified atmosphere. AnaeroPack-Anaero (MGC, Japan) and anaerobic jar (MGC, Japan) were used for cell hypoxia as previously described [13]. Briefly, culture medium was changed into DMEM (Gibco, USA) without glucose and serum before hypoxia. Ad-HGF (12.5, 25 or 50 MOI), HGF (80 ng/ml), HGF receptor inhibitor SU11274 (10 μM) or NF-κB inhibitor PDTC (20 μM) was added into medium before hypoxia in different groups. H9c2 cells, anaerobic indicator and AnaeroPack were placed into the anaerobic jar and the jar lid was immediately closed. After about thirty minutes, the oxygen concentration decreased to less than 0.1%. The whole anoxic process was performed at 37°C.

Transmission electron microscope

After treatment, the cells were collected using 0.25% trypsin (containing 0.02% EDTA) digestion, washed in fresh PBS (pH=7.4), and fixed with 2.5% glutaraldehyde. Cell specimens were post-fixed in buffer containing 1% OsO_4 and 1% potassium ferrocyanide, dehydrated in a series of graded ethanol solutions, and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin section (60 nm) was cut on a Reichert Ultracut-S microtome, placed onto copper grids, stained with uranyl acetate and lead citrate, and observed on a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

Ad-mRFP-GFP-LC3 adenovirus infection

Cells were cultured on slides in 24-well plates and allowed to reach about 60% confluence at the time of transfection. Ad-mRFP-GFP-LC3 adenoviral vectors infection was performed according to the manufacturer's instructions (HanBio, Shanghai, China). Cells were incubated in growth medium with the adenoviruses at a MOI of 100 for 6 h at 37°C, and then grown in new medium for another 24 h at 37°C. After infection, cells were treated with different reagents and hypoxia as legends. Autophagy was observed under LSM 5 Live DuoScan Laser Scanning Microscope (Zeiss, Oberkochen, Germany). Autophagic flux was determined by evaluating the number of GFP, RFP and merged points (dots/cells were counted).

Western blot analysis

Cells were harvested and lysed, and then protein concentration was quantified using BCA Protein Assay (Pierce, Rockford, IL, USA). Ele-

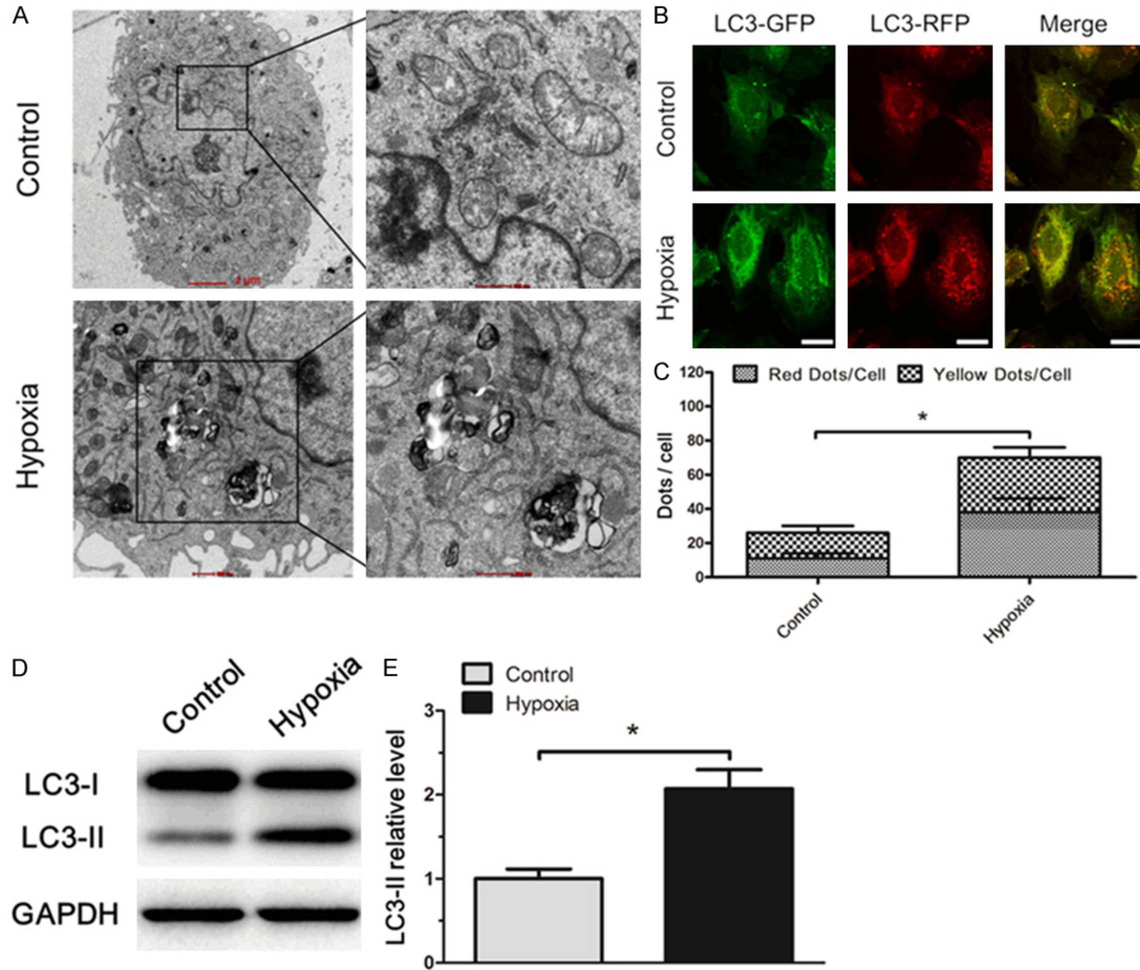

Figure 1. Hypoxia treatment induced autophagy in H9c2 cells at early stage. A: Transmission electron microscope was used to observe cellular morphology changes in control and hypoxia groups. Autophagosomes were observed in H9c2 cells after hypoxia for 3 hours. B: Laser confocal microscope analysis of H9c2 cells transiently transfecting Ad-mRFP-GFP-LC3 in H9c2 cells of hypoxia for 3 hours or no hypoxia group (scale bar, 20 μm). C: The bar graph showing the statistical analysis of gathered LC3 fluorescent dots in the indicated groups. D: Representative western blot images of LC3-I and LC3-II in control and hypoxia groups. GAPDH was used as loading control. E: The bar chart showed the quantitative analysis of the above protein levels. Data are expressed as mean ± SD, n=4, *$P<0.05$.

ctrophoresis was performed on SDS-PAGE gels. Separated proteins were transferred from the gels to PVDF membranes (Millipore, Billerica, MA, USA). Protein blots were probed with the indicated primary antibodies and appropriate secondary antibodies and protein bands were visualized using the ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad, Hercules, CA, USA). Densitometric analysis of band intensity was performed using Imagelab software (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

H9c2 cells were cultured on coverslips, and fixed in 4% paraformaldehyde after the indicated treatment. The slides were gently rinsed twice with PBS and permeabilize with 0.1% Triton X-100 for 10 minutes. The slides were rinsed twice with PBS and then blocked in 2.5% BSA in PBS for 30 minutes. Sections were incubated with p-NF-κB p65 (1:250) diluted in the blocking buffer at 4°C overnight. Prior to the addition of the secondary antibody, the slides were rinsed three times with PBS. Secondary antibodies were added in the same blocking buffer (for green light: use 1/200 dilution of fluorescein) for 1 hour in the dark, followed by another three washes in PBS. The sections were mounted with VECTASHIELD mounting medium with Diamidino-2-phenylindole (DAPI),

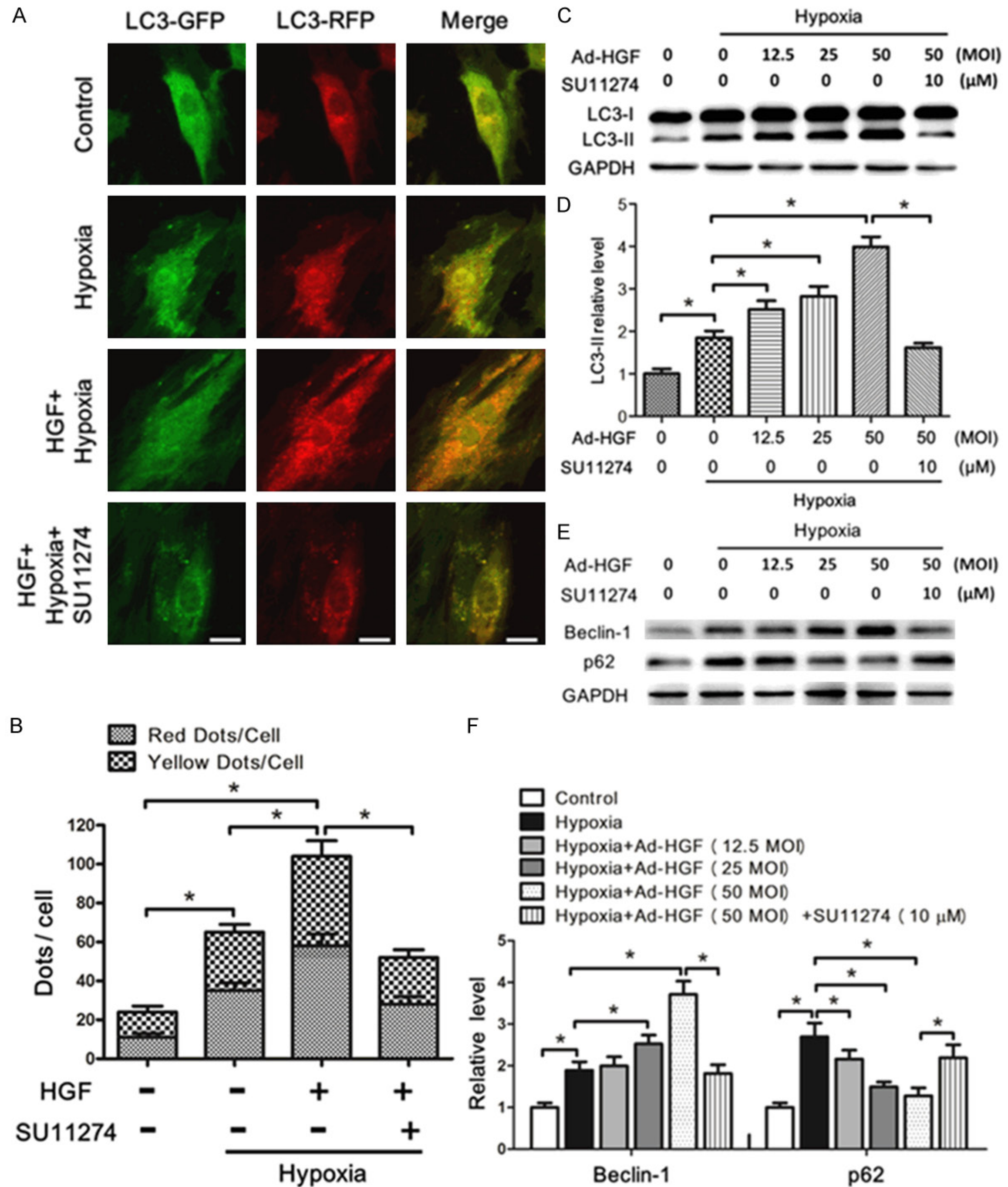

Figure 2. Ad-HGF promoted autophagy in H9c2 cells under hypoxia. A: Laser confocal microscope analysis of H9c2 cells transiently overexpressing Ad-mRFP-GFP-LC3. HGF (80 ng/mL), SU11274 (10 μM) or PBS was added into medium and hypoxia for 3 hours. Scale bar, 20 μm. B: Statistic analysis of fluorescent points in H9c2 cells. C: Western blots analysis of LC3-I and LC3-II in the indicated groups. D: Statistic analysis of LC3 II protein densitometric quantification. E: Western blot analysis of Beclin1 and p62 in H9c2 cells after hypoxia for 3 hours. F: The bar graph showing the statistical analysis of the above protein densitometric quantification. Data are expressed as mean ± SD, n=4, *P<0.05.

(Vector Laboratories, Burlingame, CA, USA), and captured images under LSM 5 Live Duo-Scan Laser Scanning Microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

All data are expressed as the mean ± SD. Statistical significance between groups was

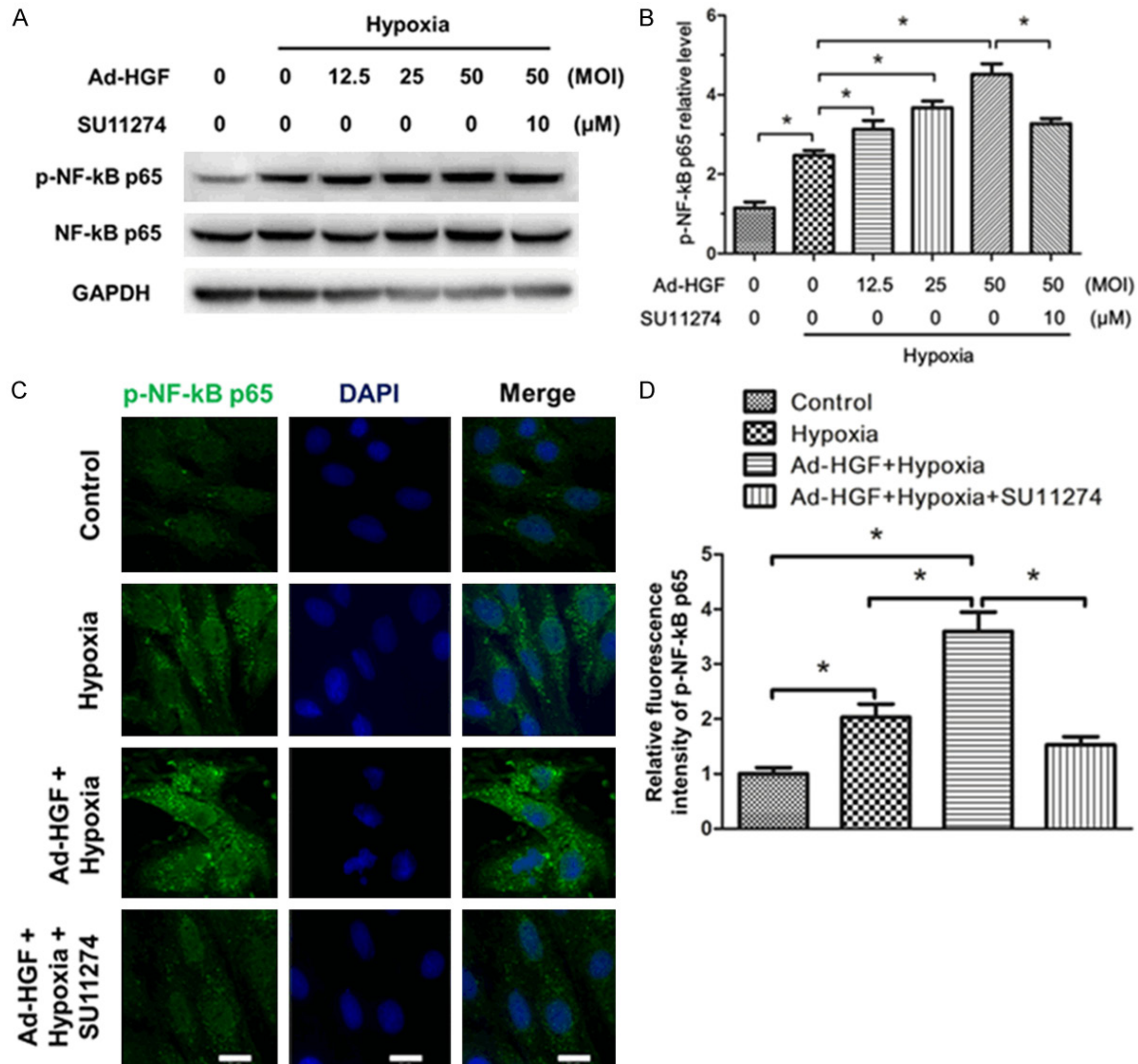

Figure 3. Ad-HGF activated NF-κB signaling in H9c2 cells under hypoxia. A: Representative western blot images of p-NF-κB p65 and NF-κB p65 in indicated groups. B: The bar graph showing the statistical analysis of the above protein densitometric quantification. C: Representative immunofluorescence images of H9c2 cells staining with p-NF-κB p65 (green) and DAPI (blue) in indicated groups. Scale bar, 20 μm. D: The bar graph showing the statistical analysis of p-NF-κB p65 relative fluorescence intensity in indicated groups. Data are expressed as mean ± SD, n=4, *P<0.05.

assessed using one-way ANOVA followed by Fisher's exact test. A value of less than 0.05 was considered statistically significant.

Results

Hypoxia treatment induced autophagy in H9c2 cells at early stage

To investigate the effect of hypoxia on H9c2 cells' autophagy, we used transmission electron microscope (TEM), Ad-mRFP-GFP-LC3 adenovirus infection and western blot to detect autophagy after hypoxia. Notably, as early as 3 hours of hypoxia, TEM detected the typical autophagosome in H9c2 cells (**Figure 1A**), which was further confirmed by the following autophagy flux observation. Only when autophagy occurs, mRFP and GFP tagged LC3 gathered together and formed fluorescence points which could be counted under fluorescence microscope. In merged images, red points represented autolysosome while yellow (merged by green and red points) represented autophagosome [13]. As expected, both autophagosome and autolysosome were significantly

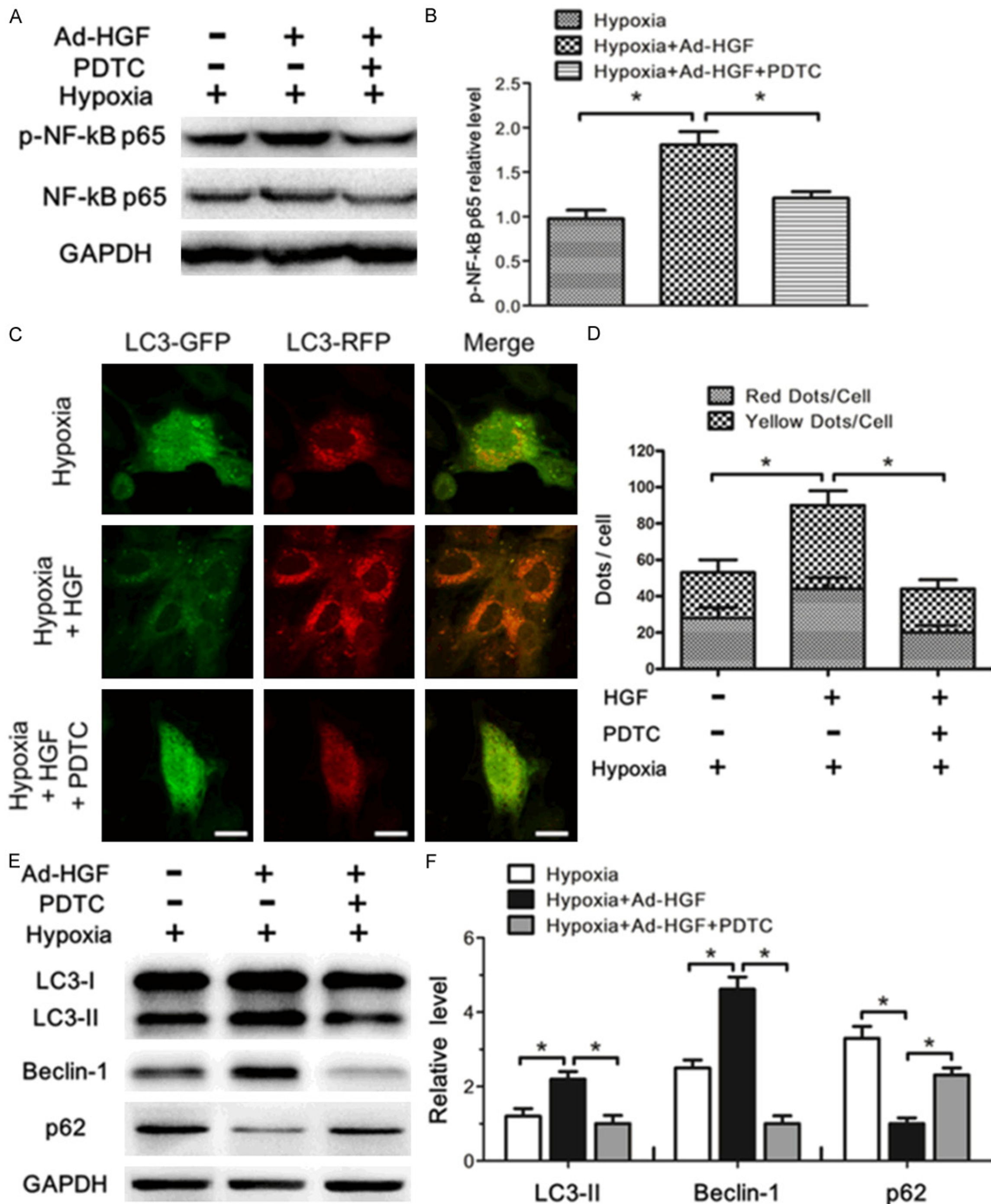

Figure 4. Activation of NF-κB by Ad-HGF resulted in upregulation of autophagy. A: Representative western blot images of p-NF-κB p65 and NF-κB p65 in indicated groups. B: Statistical analysis of the above protein densitometric quantification. C: Laser confocal microscope analysis of H9c2 cells transiently overexpressing Ad-mRFP-GFP-LC3. HGF (80 ng/mL), PDTC (20 μM) or PBS was added into medium and hypoxia for 3 hours. Scale bar, 20 μm. D: Statistic analysis of fluorescent points in H9c2 cells. E: Western blot analysis of LC3-I, LC3-II, Beclin-1 and p62 proteins. F: Protein densitometric quantification of LC3-II, Beclin-1 and p62. Data are expressed as mean ± SD, n=4, *$P<0.05$.

increased in H9c2 cells after hypoxia for 3 hours (**Figure 1B** and **1C**). LC3-II was a widely used indicator to mark autophagy [14]. The results of western blot also showed that autophagy was markedly enhanced soon after hypoxia (**Figure 1D** and **1E**).

Taken together, our data suggest hypoxia induced autophagy in H9c2 cells at early stage which is consistent with previous report that autophagy happens at early stage of cardiomyocyte ischemia which is adaptive and protectable to cells [5]. In addition, 3 hours of hypoxia for H9c2 cells was chosen to study autophagy in the following experiments.

HGF overexpression promoted autophagy in H9c2 cells under hypoxia

Our previous study has reported adenoviral vector carrying the HGF gene (Ad-HGF) mediated HGF overexpression in myocardial tissue after infarction exerted protective effects on the heart [9]. Here, we explored the effect of Ad-HGF on autophagy in myocardial cell line, H9c2 cell under hypoxia. Interestingly, immunofluorescence results revealed that HGF significantly increased cell autophagy at early stage, which was evidenced by markedly increased fluorescence points, while HGF receptor inhibitor, SU11274 had the opposite effects (**Figure 2A** and **2B**).

The appropriate MOI of Ad-HGF to infect the H9c2 cells had been explored and we chose the 12.5, 25, 50 MOI of Ad-HGF for the following experiments. Ad-HGF (12.5, 25, 50 MOI) gradually increased the HGF and p-Met protein levels in H9c2 cells under hypoxia which could be blocked by SU11274. Our results demonstrated Ad-HGF worked well at the selected MOIs (Supplementary Figure 1).

Moreover, Ad-HGF treatment dose-dependently increased LC3-II protein level in H9c2 cell under hypoxia which confirmed the results above (**Figure 2C** and **2D**). Beclin1 plays a promoting role in both autophagosome formation and autolysosome fusion [15]. The further western blot results suggested HGF overexpression enhanced Beclin1 protein level in a dose-dependent fashion, which account for the role of Ad-HGF to promote autophagy (**Figure 2E** and **2F**). Co-accumulation of p62 and protein aggresomes in the heart detrimentally affects cardiomyocytes and promotes heart failure [16]. Intriguingly, Ad-HGF dramatically attenuated p62 level in H9c2 cells which can be reversed by SU11274 (**Figure 2E** and **2F**).

Collectively, our results demonstrated Ad-HGF promoted autophagy in H9c2 cells under hypoxia; however, its mechanism needs further research.

Ad-HGF activated NF-κB signaling in H9c2 cell under hypoxia

NF-κB-mediated induction of autophagy in cardiac ischemia/reperfusion injury has been reported [12], thus we further explored the effect of HGF on the activity of NF-κB. The results showed that overexpression of HGF significantly upregulated the protein expression of phosphorylated NF-κB p65 compared with hypoxia-injured cells, while HGF receptor inhibitor had the opposite effect (**Figure 3A** and **3B**). Nuclear transfer is one of the characteristics of p65 activation. To visualize the location and activity of p65, we performed immunofluorescence staining against p-NF-κB p65. Interestingly, Ad-HGF significantly increased not only the nuclear but also the cytoplasmic p-NF-κB p65 expression in H9c2 cell under hypoxia (**Figure 3C** and **3D**). These findings demonstrated that HGF overexpression activates the NF-κB signaling pathway.

Activation of NF-κB by Ad-HGF resulted in upregulation of autophagy

To further explore whether activation of NF-κB by HGF is responsible for the activation of autophagy in H9c2 cells under hypoxia, we utilized a NF-κB specific inhibitor, PDTC in H9c2 cells' hypoxia model to clarify the association. Firstly, we observed PDTC treatment significantly suppressed the p-NF-κB p65 expression in H9c2 cells induced by Ad-HGF, which indicated PDTC inhibited the NF-κB signaling (**Figure 4A** and **4B**). Next, PDTC inhibited Ad-HGF-induced autophagy process which was evidenced by significantly decreased LC3 fluorescence dots compared with HGF group, which manifested PDTC-mediated NF-κB signaling blocking attenuated HGF-induced autophagy in H9c2 cells under hypoxia (**Figure 4C** and **4D**). Moreover, the western blot results revealed PDTC intervention markedly decreased LC3-II and Beclin1 protein levels and significantly increased p62 protein level when compared with Ad-HGF-treated group (**Figure 4E** and **4F**).

Combined the data above, we demonstrated that activation of NF-κB signaling by Ad-HGF resulted in the activation of autophagy in H9c2 cells under hypoxia.

Discussion

Our findings demonstrated that adenoviral vector mediated HGF overexpression promoted autophagy in H9c2 cells under hypoxia, which is mediated by activating NF-κB signaling.

Autophagy is an intracellular bulk degradation process whereby cytosolic, long-lived proteins and organelles are degraded and recycled [1]. Autophagy occurs at basal levels but can be further induced by stresses, such as nutrient depletion, myocardial ischemia, ischemia/reperfusion and MI, in which autophagy plays a compensatory role by scavenging damaged mitochondria and protein aggregates and alleviating energy loss [16]. Methods of glucose deprivation, serum starvation or growth factor deprivation etc. have been reported to induce autophagy *in vitro* [17]. To mimic the pathological process of MI *in vitro*, we induced autophagy by treating rat myocardial cell line, H9c2 cell, with serum-free and glucose-free medium in a hypoxia chamber. Intriguingly, as early as 3 hours of hypoxia, occurrence of autophagy was observed by TEM and further verified by increased mGFP-RFP-LC3 dots and LC3-II protein level, which implies autophagy is sensitive and can be regarded as a rapid response to hypoxia insult. The results suggested that the measure we took to mimic MI *in vitro* was effective and identified autophagy during this process. Moreover, acute induction of autophagy has been regarded as a novel strategy for cardioprotection [18].

Next, we found adenoviral vector mediated HGF overexpression in H9c2 cells dose-dependently promoted autophagy under hypoxia, which could be blocked by the specific HGF receptor inhibitor, SU11274. Ad-HGF treatment significantly increased LC3-II and Beclin1 protein levels and markedly decreased p62 protein level. In addition, previous studies have suggested that Beclin1 plays a promoting role in both autophagosome formation and autolysosome fusion [19]. And p62 is often found to co-localize with aggresomes including accumulation of damaged proteins and organelles in failing heart, which can be scavenged and degraded by autophagy [16]. Thus, these data imply the protective role of adaptable autophagy in hypoxia-injured H9c2 cells induced by Ad-HGF. On the contrary, Gallo et al. suggested agonist antibodies could activate the c-Met receptor and attenuate cobalt chloride-induced autophagy in cardiomyocytes [20]. These contradictory phenomena might result from different hypoxia methods. In their study, cobalt chloride was employed to imitate cell chronic hypoxia, but we used serum-glucose free medium and anaerobic jar to mimic hypoxia for H9c2 cells.

NF-κB is known to regulate multiple cellular processes, and several molecules involved in autophagy are dependent on NF-κB signaling. The relationship between NF-κB and autophagy is still controversial. Copetti et al. suggested that NF-κB upregulates autophagy by transactivating the autophagy-triggering Beclin1 protein [21]. Zeng et al. reported that cardiac ischemia/reperfusion injury promotes NF-κB activity and activates Beclin 1-mediated autophagy [12]. Conversely, Gao et al. reported that inhibition of NF-κB promotes autophagy via JNK signaling pathway in porcine granulosa cells [22]. Here, our data showed HGF overexpression enhanced autophagy in hypoxia-injured H9c2 cells through activating NF-κB signaling including increased p-NF-κB p65 protein level and translocation into the nucleus, while NF-κB specific inhibitor, PDTC had the opposite effect. After careful comparison and analysis, we think different cell types and interventions in various studies, as well as different molecular mechanisms involved in different diseases may be account for the difference of the relationship between NF-κB and autophagy.

HGF is a powerful pro-survival factor and has been shown to enhance cardiomyocyte survival under ischemic conditions. Studies that blocked HGF activity, either by utilizing siRNA against HGF or blocking the HGF receptor, c-met, have shown negative effects on tumor cell activity by reducing proliferation and invasion [23]. Our previous studies has found adenoviral vector mediated HGF overexpression in infarction border zone after MI improves cardiac remolding by anti-apoptosis, anti-fibrosis, promoting angiogenesis and myocardium regeneration [8, 9]. Here, we firstly reported Ad-HGF promotes autophagy in hypoxia-injured H9c2 myocardial cell line via upregulating NF-κB signaling. And It has been confirmed enhancement of autophagy can decrease infarction size after myocardial infarction and reduce adverse ventricular remodeling [18, 24].

In summary, we found that Ad-HGF treatment promoted autophagy in hypoxia-injured H9c2 cells. This protective effect of adaptable autophagy is mediated by activating NF-κB signaling, which suggests a new potential pathway of HGF protecting myocardium from hypoxia.

Acknowledgements

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Disclosure of conflict of interest

None.

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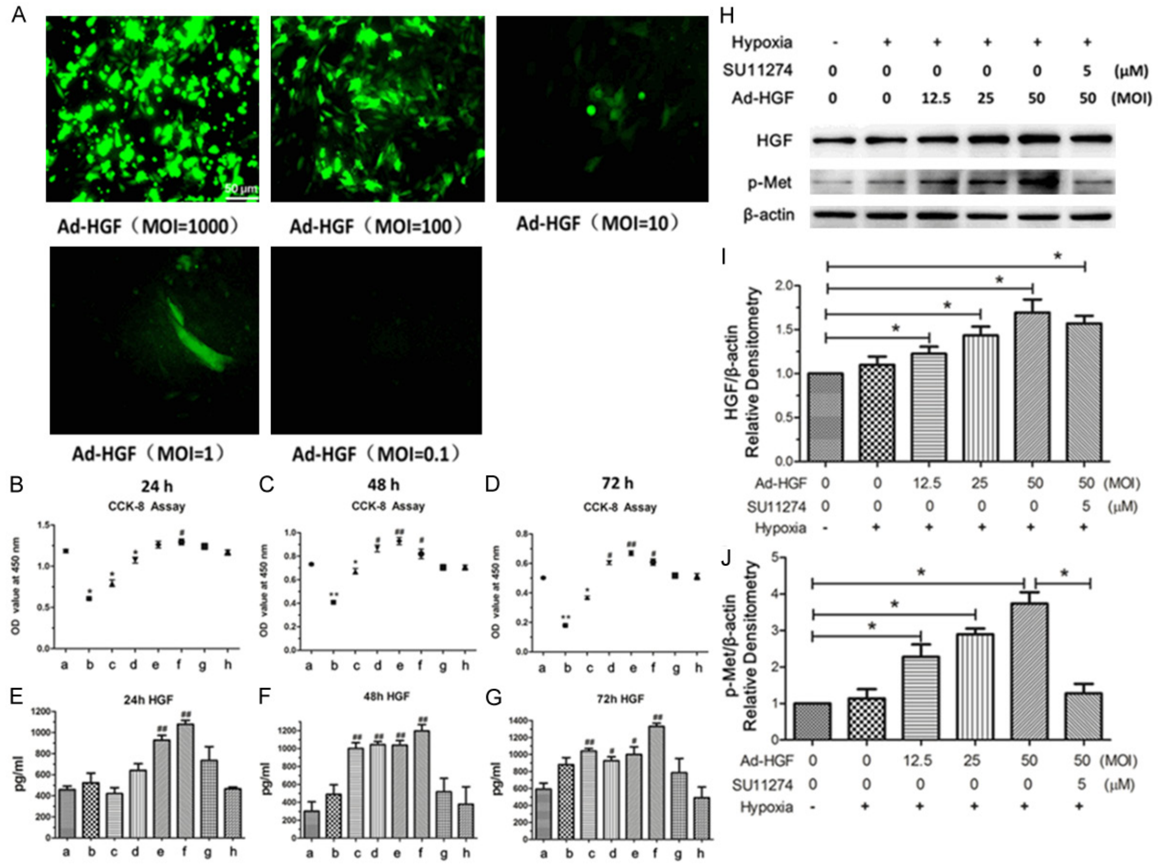

Supplementary Figure 1. Choose the appropriate multiplicity of infection (MOI) of Ad-HGF to infect the H9c2 cells. A: Different MOIs of Ad-HGF (1000, 100, 10, 1, 0.1 MOI) were used to infect the H9c2 cells under the same cell density for 24 hours and the GFP fluorescence conjugated was observed under the fluorescence microscope. The infection efficiency of Ad-HGF (1000 or 100 MOI) was high, but the number of dead cells after infection increased. B-D: CCK-8 assay was further used to assess the cell activity after infection of Ad-HGF with various MOIs. a. control, b. MOI=1000, c. MOI=200, d. MOI=100, e. MOI=50, f. MOI=25, g. MOI=12.5, h. MOI=6.125. The CCK-8 assays of infection for 24, 48 or 72 hours showed the cell activity with infection of Ad-HGF (1000, 200, 100 MOI) was significantly decreased when compared with the control, while the cell activity with infection of Ad-HGF (50, 25, 12.5, 6.125 MOI) not. E-G: The HGF levels in supernatant after Ad-HGF (1000, 200, 100, 50, 25, 12.5, 6.125 MOI) infection for 24, 48 or 72 hours were determined by the ELISA assay. Combined the data above, we selected the 50, 25, 12.5 MOI of Ad-HGF for the following experiments. Data are expressed as mean ± SD, n=3, *P<0.05 vs. the control, **P<0.01 vs. the control, #P<0.05 vs. the control, ##P<0.01 vs. the control. H: Western blot analyzed the protein levels of HGF and p-Met in H9c2 cells under hypoxia after Ad-HGF (12.5, 25, 50 MOI) and SU11274 (5 μM) treatment. β-actin was used as loading control. I, J: The bar graphs showed the statistical analysis of the above protein levels. Data are shown as mean ± SD, n=3, *P<0.05.